

# Effects of Qing Hua Chang Yin on lipopolysaccharide-induced intestinal epithelial tight junction injury in Caco-2 cells

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**Abstract.** Disruption of the intestinal mucosal barrier integrity is a pathogenic process in inflammatory bowel disease (IBD) development, and is therefore considered a drug discovery target for IBD. The well-known traditional Chinese formulation Qing Hua Chang Yin (QHCY) has been suggested as a potential therapeutic agent for the treatment of ulcerative colitis. However, the possible underlying molecular mechanisms regarding its therapeutic effect remain unclear. Consequently, the present study investigated the effects of QHCY on lipopolysaccharide (LPS)-induced loss of intestinal epithelial barrier integrity *in vitro* using the Caco-2 cell model of intestinal epithelium. QHCY reversed the LPS-induced decrease in transepithelial electrical resistance and significantly alleviated the increased fluorescently-labeled dextran 4 flux caused by LPS. Moreover, QHCY upregulated the mRNA and protein expression levels of occludin, zona occludens-1 and claudin-1 in LPS-exposed Caco-2 cells. In conclusion, QHCY was able to protect intestinal epithelial barrier integrity following an inflammatory insult; the protective effects of QHCY may be mediated by modulation of the expression of tight junction proteins.

## Introduction

Inflammatory bowel disease (IBD) most commonly refers to ulcerative colitis and Crohn's disease, which are conditions characterized by chronic gastrointestinal tract inflammation (1). IBD has a multifactorial etiology that involves the interplay of environmental, genetic and immunological factors (2). Its common pathogenic feature is disruption of the integrity of the intestinal epithelial barrier (3). Under normal conditions, several integral cellular proteins that maintain robust intercellular connections between epithelial cells support the intestinal mucosal barrier (4). The barrier is mainly composed of intercellular junctional complexes (5-7), which consist of tight junction (TJ) proteins (occludin and claudin-1) interacting with the central protein zona occludens (ZO)-1. TJ disruption leads to disturbances in the paracellular barrier and an increase in intestinal epithelial paracellular permeability. This alteration in permeability causes potential harmful antigens and luminal bacteria to penetrate the intestine, resulting in the initiation and acceleration of the mucosal inflammation in IBD (8-11). Thus, therapies that attenuate intestinal barrier dysfunction could effectively treat IBD (10-12).

Natural products commonly used in Traditional Chinese Medicine (TCM) have gained an increased medical interest worldwide, due to their potent anti-inflammatory role (13-15). One well-known traditional Chinese formula is Qing Hua Chang Yin (QHCY), which is composed of *Coptis chinensis* Franch, *Herba et Gemma Agrimoniae*, *Radix Sanguisorbae*, *Magnolia officinalis*, *Radix Paeoniae Rubra*, *Elettaria cardamomum*, *Semen Coicis*, *Artemisia capillaris* Thunb, *Semen Dolichoris Album*, *Herba Eupatorii Fortunei* and *Poria cocos*. In TCM, QHCY is considered beneficial in the treatment of UC; therefore, QHCY has been used in the management of UC for several years in China (16-21).

In mice, QHCY has been reported to alleviate the clinical and histological manifestations of dextran sulfate sodium-induced colitis (22-24); this effect was revealed to be partly mediated by a reduction in inflammatory cytokine release via the TLR4/NF- $\kappa$ B and IL-6/STAT3 signaling pathways. However, to the best of our knowledge, the effect

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**Abbreviations:** IBD, inflammatory bowel disease; QHCY, Qing Hua Chang Yin; TJ, tight junction; UC, ulcerative colitis; TCM, Traditional Chinese Medicine

**Key words:** Qing Hua Chang Yin, Traditional Chinese Medicine, ulcerative colitis, tight junction

of QHCY on intestinal epithelial barrier function is unknown. To elucidate the therapeutic mechanism underlying the effects of QHCY, the present study investigated the *in vitro* effects of QHCY on the intestinal epithelial barrier.

## Materials and methods

**Materials and reagents.** Dulbecco's modified Eagle's medium (DMEM; cat. no. C11995500BT), fetal bovine serum (FBS; cat. no. 10091148), penicillin-streptomycin (cat. no. 15070063) and trypsin-ethylenediaminetetraacetic acid (cat. no. 25200072) were purchased from Gibco; Thermo Fisher Scientific, Inc. Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5; cat. no. L6529) and fluorescein isothiocyanate-dextran (FITC-dextran 4: FD4; cat. no. FD4) were acquired from Sigma-Aldrich; Merck KGaA. The M-PER Mammalian Protein Extraction Reagent (cat. no. 78501) and BCA assay kit (cat. no. 23227) were purchased from Thermo Fisher Scientific, Inc. Anti-ZO-1 (cat. no. GTX108592) was obtained from GeneTex, Inc., and anti-occludin (cat. no. 13409-1-AP) and anti-claudin-1 (cat. no. 13050-1-AP) antibodies were obtained from Proteintech Group, Inc. Moreover, anti- $\beta$ -actin antibody (cat. no. 4970) and horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. 7074) were acquired from Cell Signaling Technology, Inc. The human TNF- $\alpha$  ELISA kit (cat. no. 430204) was obtained from BioLegend, Inc. RNAiso plus reagent and the PrimeScriptRT reagent kit were obtained from Takara Biotechnology Co., Ltd. Unless otherwise noted, all other reagents were obtained from Sigma-Aldrich; Merck KGaA.

**QHCY preparation.** QHCY was prepared as previously described (23). Briefly, the following dehydrated amounts of each component were used in the preparation: 33 g *Coptis chinensis* Franch, 220 g Herba et Gemma Agrimoniae, 110 g Radix Paeoniae Rubra, 100 g Radix Sanguisorbae, 110 g *Magnolia officinalis*, 56 g *Elettaria cardamomum*, 110 g Herba Eupatorii Fortunei, 110 g *Artemisia capillaris* Thunb., 110 g Semen Dolichoris Album, 220 g Semen Coicis and 220 g *Poria cocos* (obtained from the Department of Pharmacy, Second People's Hospital Affiliated to Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian, China). The mixture was extracted by boiling three times in 2 l distilled water. The extracts were filtered and concentrated by boiling to a final volume of 1 l. The stock concentration of QHCY was 1.4 g/ml.

**Cell culture.** Cells were cultured as previously described (23). Human colon cancer Caco-2 cells (cat. no. HTB37) were purchased from the American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% (v/v) FBS, glucose (1 g/l), penicillin (50 U/ml) and streptomycin (50  $\mu$ g/ml) in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. Subsequently, cells were subcultured at 85-90% confluence and differentiated into enterocyte-like cells 18-20 days later, as described previously (22,23). Fully differentiated cells were used for further experiments.

**Cell Counting Kit-8 (CCK-8) assay.** Cell viability was assessed using CCK-8 (Dojindo Technologies, Inc.). Differentiated Caco-2 cells in 96-well plates were treated with the indicated

concentrations of LPS (0-80  $\mu$ g/ml) for 24 h at 37°C. Subsequently, 10  $\mu$ l CCK-8 was added to each well and incubated for 2 h at 37°C. Absorbance was measured at 405 nm using a fluorescence plate reader (Model ELx80; BioTek Corporation).

**TNF- $\alpha$  ELISA assay.** As described previously (23), differentiated Caco-2 cells in 24-well plates were incubated with QHCY (10 and 50  $\mu$ g/ml) for 1 h before stimulation with LPS (1  $\mu$ g/ml) for 24 h at 37°C. Subsequently, the supernatants were collected by centrifuging the cell culture medium at 3,000 x g for 10 min at room temperature. Using a human TNF- $\alpha$  ELISA kit, the production of TNF- $\alpha$  from Caco-2 cells was measured, according to the manufacturer's instructions. Absorbance was read at 450 nm using a fluorescence plate reader. All samples were assessed in triplicate.

**Transepithelial electrical resistance (TEER) measurement.** Caco-2 cells (5x10<sup>4</sup> cells/well) were seeded in the upper chamber of 24-well plates containing Transwell inserts and cultured for 18-20 days before experimentation; the culture medium was changed every other day. To determine TJ formation, the TEER of the Caco-2 cell monolayer was measured using a Millicell ERS (EMD Millipore). For the subsequent experiments, monolayers with TEER between 400 and 500  $\Omega$  cm<sup>2</sup> were used, and were treated with QHCY (10 and 50  $\mu$ g/ml) for 1 h before LPS (1  $\mu$ g/ml) stimulation for 24 h at 37°C. TEER was measured before and after treatment. Moreover, triplet cell monolayers were assessed for each experimental group. TEER changes during experimental conditions were calculated as the percentage of baseline levels.

**Measurement of permeability.** To evaluate paracellular permeability, the fluorescently-labeled dextran 4 (FD4; cat. no. FD4; Sigma-Aldrich; Merck KGaA) flux from apical to basolateral was measured as previously described, with minor modifications (25). Briefly, Caco-2 cells (5x10<sup>4</sup> cells/well) were seeded in the upper chamber of 24-well plates containing Transwell inserts and were cultured for 18-20 days before experimentation; the culture medium in the lower chamber was changed every other day. The TEER of the Caco-2 cell monolayer was measured using a Millicell ERS (EMD Millipore). For the subsequent experiments, the cell monolayers with TEER between 400 and 500  $\Omega$  cm<sup>2</sup> were used, and treated with QHCY and LPS as aforementioned. The medium was then removed, and 200  $\mu$ l FD4 (5 mg/ml) and 500  $\mu$ l PBS was added to the apical and basolateral compartments of each Transwell insert, respectively. After 2 h of incubation at 37°C, 100  $\mu$ l was removed from the basolateral compartment and transferred to 96-well plates. Subsequently, FD4 concentration was determined using a fluorescence plate reader at an excitation wavelength of 480/492 nm and an emission wavelength of 520/525 nm. All samples were assessed three times.

**Reverse transcription-quantitative PCR (RT-qPCR) assay.** Differentiated Caco-2 cells in 6-well plates were incubated with QHCY and LPS as aforementioned. According to the manufacturer's instructions, total cellular RNA was extracted using RNAiso plus reagent (Takara Biotechnology Co., Ltd.) and RT was conducted using the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.). The mRNA expression levels of ZO-1,

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene name	Sequences (5'-3')
ZO-1	Forward: AGCCTGCAAAGCCAGCTCA Reverse: AGTGGCCTGGATGGGTTCATAG
Claudin-1	Forward: GCATGAAGTGTATGAAGTGCTTGGA Reverse: CGATTCTATTGCCATACCATGCTG
Occludin	Forward: CTTTGGCTACGGAGGTGGCTAT Reverse: CTTTGGCTGCTCTTGGGTCTG
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC Reverse: ATGGTGGTGAAGACGCCAGT

ZO-1, zona occludens-1.

occludin and claudin-1 were determined by qPCR using SYBR green dye (Thermo Fisher Scientific, Inc.) and the ABI 7500 fast sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). An initial denaturation step was performed at 95°C for 30 sec, followed by 40 cycles at 95°C for 3 sec and annealing at 60°C for 30 sec. GAPDH served as an internal control. The sequences of primers used for qPCR are provided in Table I. The mRNA expression levels of genes of interest were quantified using  $2^{-\Delta\Delta C_q}$  analysis (26). mRNA expression levels are expressed as fold change relative to the control group.

**Western blot analysis.** Differentiated Caco-2 cells in 6-well plates were incubated with QHCY (10 and 50  $\mu\text{g}/\text{ml}$ ) for 1 h before stimulation with LPS (1  $\mu\text{g}/\text{ml}$ ) for 24 h at 37°C. A mammalian cell lysis buffer containing a phosphatase inhibitor and a protease inhibitor cocktail (EMD Millipore) (23) was used to lyse the differentiated Caco-2 cells incubated with QHCY and LPS for 24 h at 37°C. Lysed cells were then centrifuged at 12,000  $\times g$  for 15 min at 4°C and the supernatants were collected. Protein concentrations were measured using the BCA protein assay kit. Equal amounts of protein (50  $\mu\text{g}$ ) from each sample were resolved on 12% Tris-glycine gels and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with SuperBlock buffer (Thermo Fisher Scientific, Inc.) and were then incubated with primary antibodies against ZO-1 (rabbit, polyclonal; 1:1,000), occludin (rabbit, polyclonal; 1:1,000), claudin-1 (rabbit, polyclonal; 1:1,000) and  $\beta$ -actin (rabbit, polyclonal; 1:2,000) at 4°C overnight. Subsequently, membranes were incubated with the appropriate anti-rabbit IgG, HRP-linked secondary antibody (1:5,000) for 1 h at room temperature. Thereafter, the membranes were visualized using enhanced chemiluminescence using SuperSignal™ West Dura Extended Duration Substrate (cat. no. 34076; Thermo Fisher Scientific, Inc.). Images were captured using an imaging system (Bio-Rad Laboratories, Inc.). The protein levels were analyzed with ImageJ Software (National Institutes of Health).  $\beta$ -actin protein expression was used as an internal control.

**Statistical analysis.** All data were analyzed using SPSS software (22.0) for Windows (IBM Corp.). Data are presented as

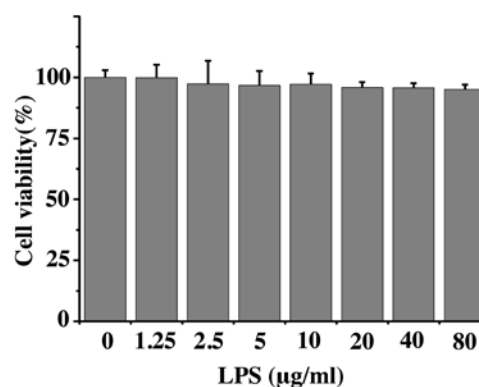


Figure 1. Effects of LPS on cell viability. Cells were treated with various LPS concentrations for 24 h and cytotoxicity was measured by Cell Counting Kit-8 assay. Data were normalized to the control group and are presented as the mean  $\pm$  SD from at least three independent experiments. LPS, lipopolysaccharide.

the mean  $\pm$  SD of at least three independent experiments. The significance of differences among groups that conformed to a normal distribution was determined by one-way ANOVA followed by Tukey's post hoc test. Two-tailed  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**LPS does not affect Caco-2 cell viability.** Caco-2 cell viability was assessed following exposure to LPS (Fig. 1); cell viability was not affected by even the highest concentration of LPS tested (80  $\mu\text{g}/\text{ml}$ ). These findings indicated that the observations made in subsequent experiments were not caused by LPS-induced cell death. A concentration of 10  $\mu\text{g}/\text{ml}$  LPS was used for all further experiments.

**QHCY inhibits the LPS-induced production of the proinflammatory cytokine TNF- $\alpha$ .** TNF- $\alpha$  has been reported to participate in the pathogenesis of various inflammatory disorders, including IBD (26). When exposed to LPS, Caco-2 cells produced increased levels of TNF- $\alpha$  compared with in the control group. To demonstrate the biological activity of the QHCY preparation, the effects of QHCY on TNF- $\alpha$  production by LPS-exposed Caco-2 cells were assessed (Fig. 2). As anticipated, LPS induced TNF- $\alpha$  secretion from the Caco-2 cells; however, QHCY significantly suppressed LPS-induced TNF- $\alpha$  secretion, which is consistent with our previous observation (24).

**QHCY inhibits inflammation-induced paracellular permeability in Caco-2 cell monolayers.** To investigate whether QHCY can preserve the intestinal epithelial barrier during inflammation, Caco-2 cell monolayers were pretreated with QHCY before LPS exposure (Fig. 3). LPS significantly decreased the TEER of the Caco-2 cell monolayers compared with that in the control group (control,  $100 \pm 2.3\%$ ; LPS,  $39.8 \pm 7.5\%$ ), indicating an increase in permeability. However, pretreatment with QHCY ameliorated the LPS-induced decrease in TEER, demonstrating that the normal barrier function was protected by QHCY treatment (10  $\mu\text{g}/\text{ml}$  QHCY,  $78.0 \pm 6.0\%$ ; 50  $\mu\text{g}/\text{ml}$  QHCY,  $96.4 \pm 7.6\%$ ). Moreover, as another measure of Caco-2

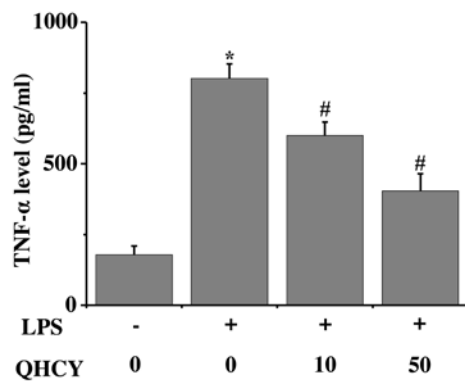


Figure 2. Effects of QHCY on TNF- $\alpha$  secretion from LPS-stimulated Caco-2 cells. TNF- $\alpha$  levels within the culture medium were determined by ELISA. Data are presented as the mean  $\pm$  SD. \*P<0.05 vs. control group; #P<0.05 vs. LPS group. LPS, lipopolysaccharide; QHCY, Qing Hua Chang Yin.

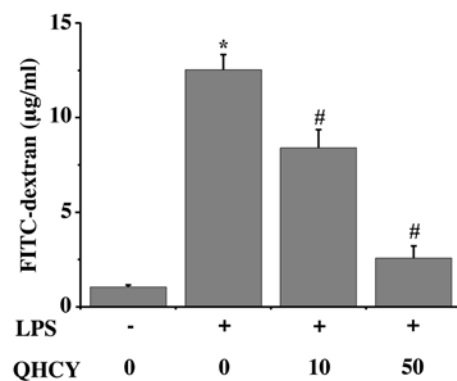


Figure 4. Effects of QHCY on FD4 flux in Caco-2 monolayers after LPS exposure. Caco-2 monolayers were treated with the indicated concentrations ( $\mu$ g/ml) of QHCY. Data are presented as the mean  $\pm$  SD. \*P<0.05 vs. control group; #P<0.05 vs. LPS group. LPS, lipopolysaccharide; QHCY, Qing Hua Chang Yin; FD4, fluorescently-labeled dextran 4.

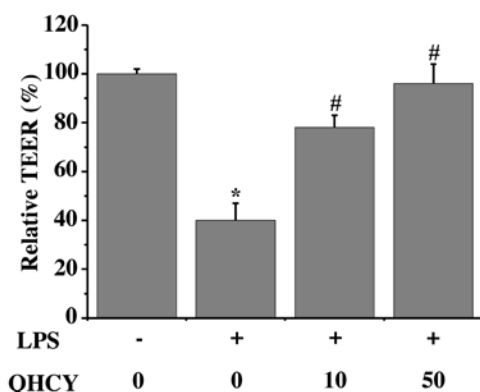


Figure 3. Effects of QHCY on relative TEER of Caco-2 monolayers following LPS exposure. Caco-2 monolayers were treated with the indicated concentrations ( $\mu$ g/ml) of QHCY. Relative TEER was determined by comparing the TEER before LPS addition to that at the end of the experimental period. Data are presented as the mean  $\pm$  SD of three replicates in three independent experiments. \*P<0.05 vs. control group; #P<0.05 vs. LPS group. LPS, lipopolysaccharide; QHCY, Qing Hua Chang Yin; TEER, transepithelial electrical resistance.

monolayer permeability, FD4 flux was measured (Fig. 4). Consistent with the TEER results, LPS significantly increased FD4 flux compared with that in the control group (control,  $1.04 \pm 0.11$   $\mu$ g/ml; LPS,  $12.52 \pm 0.80$   $\mu$ g/ml). Pretreatment with QHCY provided protection against the LPS-induced hyperpermeability of Caco-2 cell monolayers (10  $\mu$ g/ml QHCY,  $8.40 \pm 0.97$   $\mu$ g/ml; 50  $\mu$ g/ml QHCY,  $2.57 \pm 0.64$   $\mu$ g/ml). Taken together, these findings suggested that QHCY could contribute to the maintenance of mucosal barrier integrity against inflammation-induced permeability.

**QHCY prevents LPS-induced disruption of TJs in Caco-2 cell monolayers.** The present study investigated the expression levels of TJ-associated factors by RT-qPCR and western blot analyses, in order to determine the mechanisms underlying the protective effect of QHCY on epithelial cell monolayer permeability. The effects of QHCY on the mRNA expression levels of ZO-1, occludin and claudin-1 in epithelial cells were determined by RT-qPCR. As shown in Fig. 5, the mRNA expression levels of occludin (Fig. 5A), ZO-1 (Fig. 5B) and

claudin-1 (Fig. 5C) were significantly decreased after LPS treatment compared with those in the control group, whereas treatment with QHCY significantly reversed the effect of LPS on the expression levels of ZO-1, occludin and claudin-1. Similarly, the effects of QHCY on the protein expression levels of ZO-1, occludin and claudin-1 in epithelial cells were determined using western blot-ting. As shown in Fig. 6A and B, compared with in the control group, treatment with LPS induced a significant reduction in the protein expression levels of TJ-associated factors, ZO-1, occludin and claudin-1, which was reversed by the administration of QHCY. These findings suggested that preventing the reduction in the expression levels of TJ proteins may be the mechanism underlying the effects of QHCY on the LPS-induced increase in epithelial cell monolayer permeability.

## Discussion

Despite recent advances in therapy, patients with IBD still suffer from disease manifestations stemming from unremitting active inflammation. Moreover, current therapies are related to significant potential adverse reactions, such as systemic immunosuppression, headache, nausea and fatigue (27,28); therefore, alternative effective therapies for IBD are required. The anti-inflammatory effects of numerous natural products have gained considerable attention due to their relatively low toxicity (24). QHCY, which is a well-recognized traditional Chinese formula, has been used in clinical settings for treating several inflammatory disorders, including arthritis, hepatitis, cholecystitis and IBD (29). However, the mechanisms underlying the biological activity of QHCY remain to be elucidated. The barrier function of colonic mucosa is maintained by TJs and their molecular components, including ZO-1, occludin and claudin-1, and an increase in intestinal permeability is often documented in intestinal barrier dysfunction (30-32). Thus, the present study examined the effects of QHCY on cell permeability and TJ protein expression using an *in vitro* model of intestinal epithelium that has been used in similar studies (8,33-41).

Intestinal permeability serves a critical role in host defense (34). Patients with IBD exhibit increased intestinal

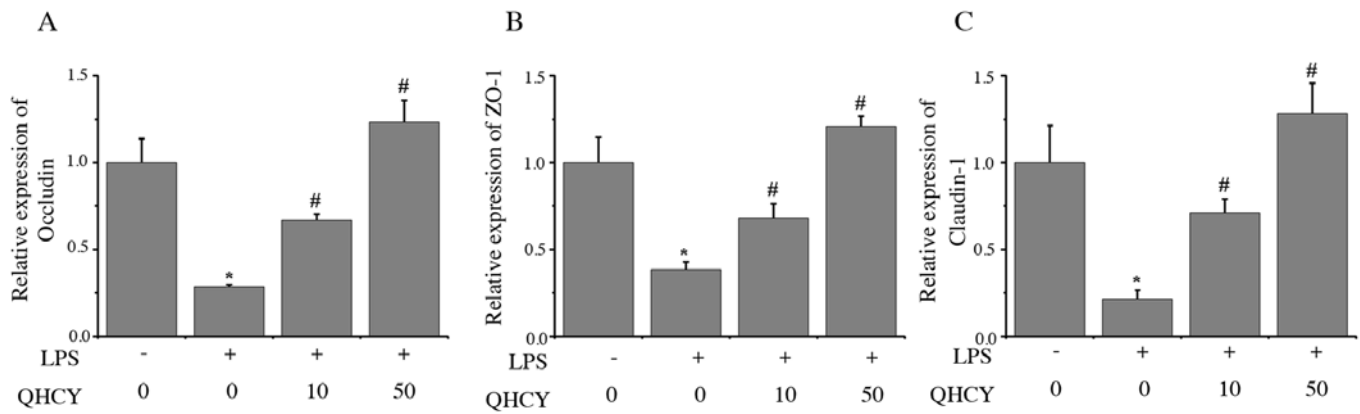


Figure 5. Effects of QHCY on the mRNA expression levels of occludin, ZO-1 and claudin-1 in LPS-exposed, differentiated Caco-2 cells treated with or without QHCY. (A) Occludin, (B) ZO-1 and (C) claudin-1 expression levels were identified by reverse transcription-quantitative PCR. GAPDH served as the internal control. Data are presented as the mean  $\pm$  SD. \* $P$ <0.05 vs. control group; # $P$ <0.05 vs. LPS group. LPS, lipopolysaccharide; QHCY, Qing Hua Chang Yin; ZO-1, zona occludens-1.

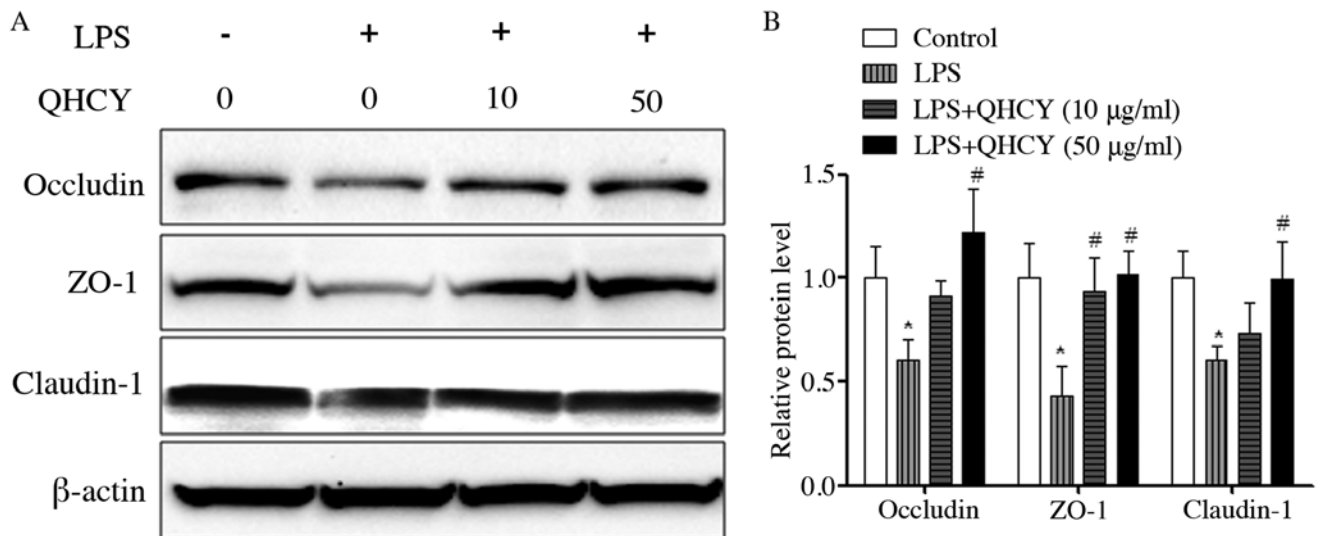


Figure 6. Effects of QHCY on the protein expression levels of occludin, ZO-1 and claudin-1 in LPS-exposed, differentiated Caco-2 cells treated with or without QHCY. (A) Occludin, ZO-1 and claudin-1 were detected by western blot analysis.  $\beta$ -actin served as the internal control. (B) Densitometric analysis. \* $P$ <0.05 vs. control group; # $P$ <0.05 vs. LPS group. LPS, lipopolysaccharide; QHCY, Qing Hua Chang Yin; ZO-1, zona occludens-1.

mucosal permeability, which is correlated with disease severity (8,35-37). In the present study, QHCY significantly alleviated LPS-induced increases in the permeability of epithelial cell monolayers. This effect was observed by both TEER and FD4 flux assays. Thus, QHCY may contribute to maintaining the integrity of the mucosal barrier during inflammatory insults.

The intestinal epithelial barrier consists of epithelial cells joined together by intercellular junctional complexes, which include the TJ proteins ZO-1, occludin and claudin-1 (5). These proteins are crucial for barrier function maintenance, and their expression has been shown to be reduced in response to inflammatory states, including in IBD (36-39). In IBD, claudin-1 expression was significantly decreased (42). As previously demonstrated that QHCY significantly reversed the DSS-induced downregulation of ZO-1, occludin, and claudin-1 in mRNA and protein levels in the mice (30). The present study revealed that LPS markedly suppressed the

expression of ZO-1, occludin and claudin-1 at both the mRNA and protein expression levels in IEC, whereas QHCY significantly reversed this effect. Therefore, QHCY may regulate TJ expression to maintain mucosal barrier integrity. Possibly, the effects of QHCY observed on the expression levels of TJ proteins were indirect and caused by TNF- $\alpha$  activity suppression, since QHCY could inhibit the LPS-induced production of the proinflammatory cytokine TNF- $\alpha$ . Notably, QHCY may exert potential anti-inflammatory effects and could be considered a useful therapy for the treatment of IBD.

In conclusion, QHCY reduced the LPS-induced secretion of TNF- $\alpha$  and reversed the increase in permeability of Caco-2 cell monolayers, thereby preserving the expression levels of ZO-1, occludin and claudin-1. Therefore, QHCY may be considered a potential therapeutic agent for the treatment of IBD, due to its direct suppressive effect on the secretion of proinflammatory cytokines and the maintenance of epithelial barrier function. However, further investigations should be

conducted to determine the underlying potential mechanism of QHCY treatment for IBD.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XK, TJS and JP acquired funding for the research. WF, PZ, AS, YL, SS and XK conceived and designed the experiments. WF, PZ, AS, LL, HC, SS and YC performed the experiments. WF, PZ, TS and AS analyzed the data. YC, TJS and JP acquired, interpreted the data and confirmed the authenticity of the raw data associated with the preparation of the manuscript. JP, TJS and SS wrote the manuscript. JP, YL, TJS and XK checked the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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